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(54) **Gene encoding asymmetrically active esterase.**

(57) An isolated gene encoding an esterase capable of asymmetrically hydrolysing an ester of chrysanthemumic acid or its derivative to give an intermediate useful for the production of pharmaceutically and/or agriculturally useful compounds, expression plasmids containing said gene, microorganisms transformed with said expression plasmids, and the production of the esterase by culturing said transformants.

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This invention relates to an isolated gene encoding an esterase originated from a microorganism. More particularly, it relates to an isolated gene (or an isolated DNA) encoding an esterase useful for the asymmetric hydrolysis, expression plasmids containing said DNA, microorganisms transformed with said expression plasmids, and the production of the esterase by culturing said transformants.

5 The recombinant esterase obtained according to the present invention can be used for the preparation of an optically active compound useful as an intermediate for clinically or agriculturally important substances. Examples of optically active compounds which can be produced by means of the esterase of the invention include an optically active chrysanthemumic acid (KCA; 2,2-dimethyl-3-isobutenylcyclopropane-1-carboxylic acid), i.e., (+)-trans-KCA, useful as an acidic part for the production of pyrethroid insecticides.

10 Medically and/or agriculturally useful compounds, as well as their intermediates, often show complicated structure, which leads to the hardness of synthesis thereof by conventional organic synthetic methods. It should be more difficult when the desired compound is optically active. For example, pyrethrins, which can be produced from KCA, possess three asymmetric carbons and their insecticidal activity varies from one stereoisomer to another. Therefore, it has been needed to establish methods for preparing KCA or KCA derivative of a desired structure for the preparation of desired stereoisomer of pyrethrins. However, the complexity of the structure prevented the development of such methods.

15 Recently, a bioreactor-system which utilizes an enzyme derived from organisms as a catalyst has been developed. This system takes advantage of the rigid stereospecificity of enzymes. Examples of enzymes employed in such reactions include an esterase derived from pig liver (Laumen et al, Tetrahedron Lett. 26:407-410 (1985); and Wang et al (J.Am.Chem.Soc. 106: 3695 (1984)). Microorganism-derived enzymes have also been used for the same purpose. For example, an esterase derived from *Bacillus subtilis* NRRL-B-558 was used for the synthesis of cephalosporin derivatives (Appl Microbiol. 30: 413-419 (1975)). However, the isolation and purification of enzyme from an organism in sufficient amount is not easy. Furthermore, the enzymes derived from mammal tissue are usually unstable (like PLE), hard to handle and uneconomical because of the limited supply, and therefore are often unsuitable for the industrial application. When an organism-derived hydrolytic enzyme such as esterase, lipase or the like is used as a bioreactor for the production of an optically active compound, it can catalyze the following illustrative reactions: (a) stereoselective hydrolysis of a racemic compound; (b) site selective hydrolysis of a pro-chiral compound which gives rise to a chiral compound; or (c) stereoselective ester-formation as the reverse reaction of the above (a) or (b).

20 In order to apply a microorganism-derived esterase to the stereoselective hydrolysis of a racemic ester, it is necessary to search into a lot of naturally occurring microorganisms to select a particular strain capable of producing a desired enzyme with a high specific activity, culture the same, and isolate and purify the produced enzyme, if desired. However, these processes generally involve many problems such as difficulty of cultivation, inadequate activity, poor efficiency, low productivity and the like. These facts made it difficult to proceed the industrial production of an optically active compound by means of a cultured microorganism or a purified enzyme therefrom. Thus, it has been needed to obtain a sufficient amounts of an esterase broadly employable for the organic synthesis of optically active compounds such as KCA. In this regard, bacterial esterase are especially useful because many kinds of biologically active enzymes can be synthesized by means of recombinant DNA technology using bacterial cells as hosts.

25 The present inventors, under these circumstances, had searched into various microorganisms and have found that a strain of *Arthrobacter globiformis*, designated as IFO-12958, produces an esterase having a high stereoselectivity to various kinds of substrates and disclosed (Japanese Patent Publication (KOKAI) No. 181788/1989). However, owing to the low expression of the esterase, *A. globiformis* IFO-12958 could not give sufficient hydrolytic efficiency and was not suited for the industrial application. In the course of further investigation, the inventors isolated the gene encoding the esterase from SC-6-98-28 (FERM BP-3618), which produces the esterase with an excellent property.

30 In order to establish the production of an esterase having stereoselective activity by means of recombinant DNA technology, the inventors have isolated and purified a peptide having an esterase activity from SC-6-98-28, cloned said gene and determined the nucleotide sequence of the gene encoding the esterase.

35 For the purpose of the invention as is herein disclosed, when the term "gene" or "DNA" are used to express a structural gene or DNA encoding an esterase, it means an isolated gene or DNA encoding esterase originated from *A. globiformis* SC-6-98-28.

40 The DNA of the invention can be transformed into a host cell such as a procaryotic cell to obtain a transformant capable of producing a recombinant esterase abundantly. The transformed cells, when cultured in an appropriate medium, give a cultured broth having significantly elevated catalytic activity of the esterase. Thus obtained cultured broth or processed material thereof, or purified esterase therefrom can

be used as a bioreactor in the asymmetric hydrolysis of a precursor compound to give a compound having a desired structure.

Once the gene has been identified and cloned, the preparation of microorganisms capable of producing the esterase and the production of said esterase can be easily accomplished using known recombinant DNA technology, which comprises, for example, constructing expression plasmids encoding said gene and transforming a microorganism with said expression plasmids, cultivating the transformant in a medium under a suitable conditions for the expression of the esterase, and isolating the product having the esterase activity, if desired.

Thus, the first object of the invention is to provide an isolated gene encoding an esterase capable of catalyzing a stereoselective hydrolysis.

The gene of the invention preferably encodes an amino acid sequence shown by the SEQ ID No.1 in the accompanying Sequence Listing, more preferably, it has the nucleotide sequence shown by the SEQ ID No.2.

The esterase-encoding gene of the invention can be obtained from a microorganism which produces a desired esterase such as *A. globiformis* SC-6-98-28. Thus, the cloning of a gene can be accomplished by constructing a genomic library from total DNA obtained from chromosomal DNA of a microorganism such as *A. globiformis* SC-6-98-28 according to a conventional technique, probing said library, and cloning a DNA encoding esterase as will be further explained below. In the illustrative example, the cloning was carried out conventionally by preparing a genomic library by isolating chromosomal DNA from SC-6-98-28, digesting the total DNA with a restriction enzyme and cloning the obtained fragments into a phage vector  $\lambda$ gt11 or a plasmid vector pUC19, transfecting the phage or transforming the competent *E. coli* JM cells with the mixture of the cloned fragments, and screening the plaques or colonies obtained.

Selection of DNA can be conducted by any of known methods such as immunometric assay using anti-esterase antibody, hybridization using synthetic DNA probes corresponding to partial amino acid sequences of a purified peptide, or screening the clones on the basis of the esterase activity. When only a part of the DNA fragment encoding the esterase is obtained, the remaining part of DNA, and consequently the full length of esterase encoding DNA, can be obtained using said fragment as a probe. In the following Example, a part of a positive clone pK-12 was used for screening pEH16, which was followed by the construction of a clone pAGE-1 encoding the entire coding region of the esterase. Thus obtained gene preferably contains a nucleotide sequence encoding an amino acid sequence shown by the SEQ ID No.1. More preferably, the gene has the nucleotide sequence shown by the SEQ ID No.2.

The DNA encoding esterase is then used to construct expression plasmids which enables a microorganism to produce the esterase using the known recombinant technology.

Thus, the second object of the invention is to provide expression plasmids containing a nucleotide sequence encoding an esterase.

Preferably, expression vectors which can be used in the invention contain sequences necessary for the replication in a host cell and are autonomously replicable. It is also preferable that the vectors contain selectable marker(s) and can be easily selected from the untransformed cells. Many vectors and restriction enzymes used for the digestion of given DNA are obtainable from commercial sources and the operations are well-known to those skilled in the art. For the purpose of the invention, it is preferable to use vectors functional in *E. coli*. Although it is in no way limited to the use of a particular vector, for the expression in *E. coli*, vectors containing promoters such as *lac*, *tac*, *trp* and the like are preferable. Such expression vectors are conveniently obtained as promoter cartridges from Pharmacia PL, Inc. Examples of especially preferred expression plasmids of the invention are PAGE-201, PAGE-202 and PAGE-203 which contain the gene shown by the above-mentioned nucleotide sequence.

The third object of the invention is to provide a microorganism transformed by an expression plasmid of the invention and capable of producing an esterase having the above-mentioned activity. Examples of suitable host cells include various eucaryotic and procaryotic cells such as *E. coli*, *Bacillus subtilis*, lactic acid bacterium and fungi. Preferred host cell is *E. coli*. Examples of preferred microorganisms are *Escherichia coli* JM109 (PAGE-201), *E. coli* JM109 (PAGE-202), *E. coli* JM109 (PAGE-203), *E. coli* JM105 (PAGE-201), *E. coli* JM105 (PAGE-202) and *E. coli* JM105 (PAGE-203). The host cell can be transformed with an expression plasmid of the invention and cultivated using any of the well known procedures in the art to give a cultured broth comprising esterase-producing cells. In the working example as mentioned below, illustrative expression plasmids pAGE-201, 202 and 203 containing the DNA encoding esterase under the control of *tac* promotor were constructed and used to transform *E. coli* JM109 or JM105. The resultant transformants expressed products having esterase activity to a high extent after cultivation. The cultured broth containing the esterase activity is then subjected to centrifugation or filtration to separate the supernatant.

E. coli host cells harboring either of expression plasmids of the invention were grown in M9 medium at 37 °C using IPTG as an inducer. A part of the harvested cells, when assayed by SDS-PAGE, gave a main band at 40,000, showing that the cell expressed desired esterase abundantly.

The esterase activity usually exists in the transformed cell and therefore cells separated from the cultured broth by filtration or centrifugation can be used in the hydrolytic reactions. However, preparations which are usually obtained from the cultured cells in conventional manners are also useful. Examples of such preparations include dried cells, cell-free extract, enzyme solution, immobilized cells or enzyme using an appropriate solid support.

Thus, the present invention further provides a method for preparing an esterase by culturing a transformant of the invention in an aqueous nutrient medium containing assimilable carbon or nitrogen sources under aerobic conditions.

When the resultant preparations are used for the asymmetric hydrolysis, the preparation is contacted with a compound to be hydrolysed to give an optically active stereoisomer useful in the pharmaceutical and/or agricultural fields. In the following Examples, an racemic ethyl ester of KCA (referred to as KCE) was hydrolyzed to give (+)-trans KCA exclusively, which is useful as an intermediate for the production of effective pyretyroid insecticides. According to the present invention, it is possible to produce plenty of esterase useful as a bioreactor by culturing transformants.

In the accompanying Figures:

Figure 1, shows a deduced amino acid sequence of an entire 2,174 bp DNA insert in plasmid PAGE-1. The DNA sequence contains a translational region encoding esterase (nucleotides 211 - 1335) and the N-terminal sequence used as a DNA probe for the screening (nucleotides 211 - 230).

Figure 2 shows a positive clones PK-12 and pEH16 obtained by a colony hybridization and a restriction map of a plasmid PAGE-1 constructed from these clones, which contains (a) a DNA originated from *A. globiformis* and (b) the entire coding region of the esterase.

Figure 3 shows a construction strategy of the esterase expression plasmids pAGE-201, pAGE-202, and pAGE-203. Each plasmid contains a synthetic DNA (c) and a *tac* promoter (bold arrow).

Following Examples further illustrate and detail the invention disclosed, but should not be construed to limit the invention.

## Example 1

### Isolation of DNA Clone Encoding an Esterase

#### 1. Preparation of Chromosomal DNA

*Arthrobacter globiformis* SC-6-98-28 strain (FERM P-11851) was pre-cultivated in a 5 ml medium (3 .0% soluble starch, 0.7 % polypeptone, 0.5 % yeast extract, 0.5%  $\text{KH}_2\text{PO}_4$ , pH 5.0) for 24 hr at 30 °C with shaking. The culture was inoculated to a 500 ml of growth medium (6 .0% soluble starch, 1.0 % polypeptone, 0.2 % yeast extract, 0.5%  $\text{KH}_2\text{PO}_4$ , pH 5.0) and grown at 30 °C with shaking until the absorbance at 660 nm ( $\text{OD}_{660}$ ) reached to 0.25, where penicillin G was added to the final concentration of 300 units/ml culture and the cultivation was continued until the  $\text{OD}_{660}$  reached to 1.0. Cells were harvested by centrifugation and suspended into 45 ml of a mixture of 150 mM NaCl, 15 mM sodium citrate, 10 mM EDTA and 27% sucrose, and egg lysozyme was added to the final concentration of 5 mg/ml. The mixture was incubated at 37 °C for 30 min and 10 ml of 10% SDS was added thereto. After the addition of protease K to the final concentration of 200  $\mu\text{g/ml}$ , the incubation continued at 37 °C for 4 hr. The culture was extracted with an equal volume of 0.1 M Tris-saturated phenol (x2) and ether (x2). DNA was precipitated from the aqueous layer by adding 2 volumes of ethanol and recovered by winding threads of DNA on a glass rod. After dryness, the recovered nucleic acids were dissolved into 5 ml Tris-EDTA (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and treated with RNase at the final concentration of 100  $\mu\text{g/ml}$  at 37 °C for 2 hr. The mixture was extracted with an equal volume of phenol-chloroform (1:1 in volume) (x2) and DNA was precipitated from the aqueous layer by the addition of 2 volumes of cold ethanol. The resultant DNA was washed with 80% ethanol, dried and dissolved into Tris-EDTA buffer. About 5.8 mg of a chromosomal DNA was obtained.

#### 2. Screening of Genomic Library

Chromosomal DNA obtained in the above 1. was digested with *Kpn*I. Vector pUC 19 (Takara Shuzo, Japan) was digested with *Kpn*I and treated with alkaline phosphatase. The *Kpn*I-digested DNA fragments

were ligated into the KpnI site of pUC 19 using T4 DNA ligase and the ligation mixture was transformed into a competent *E. coli* K-12 JM109 (Takara Shuzo, Japan). When the *E. coli* JM109 cells containing pUC 19 are grown on LB-agar medium containing ampicillin, IPTG and X-Gal, colonies give blue color as the result of the cleavage of X-Gal by  $\beta$ -galactosidase produced in the JM 109 cells. However, the transformed *E. coli* JM109 cells which has a foreign DNA fragment inserted into the multi-cloning site of pUC 19 are grown under the same condition, colorless colonies are obtained because the transformants lack the ability to express the galactosidase activity.

Thus, white colonies were selected on the plate and subjected to the colony hybridization using synthesized DNA probes corresponding to N-terminal amino acid sequence of a purified esterase. The white colonies spread on a plate was transferred onto a nylon membrane conventionally, or inoculated onto the membrane with bamboo spits. The membrane was placed on a LB-ampicillin plate and incubated at 37 °C for several hours until colonies appeared on the membrane. DNA was extracted from the colonies by soaking (x2) the membrane into 0.5N NaOH to lyse cells, washing (x2) the membrane with 1M Tris-HCl (pH 7.5) for the neutralization. DNA was then fixed onto the membrane by drying at 80 °C under vacuum for 2 hr.

The membrane was treated at 55 °C for 1 hr in a mixture (A) comprising 6x SSC, 10 x Denhardt's reagent (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin) and then at 55 °C for 4 hr in a mixture (B) comprising 6x SSC, 1% SDS, 10 x Denhardt's reagent, 100  $\mu$ g/ml denatured salmon sperm DNA. The hybridization was performed at 55 °C for overnight in a plastic bag containing membranes, the above mixture (B) and radiolabeled probes (5 x 10<sup>5</sup> cpm/membrane) prepared by labeling the DNA mixture at the 5' terminus with [ $\gamma$ -<sup>32</sup>P] ATP and purifying by a column chromatography. After hybridization, the membranes were washed sequentially (1) at 55 °C for 15 min in 6x SSC, (2) at 55 °C for 30 min in 6x SSC, (3) at 55 °C for 30 min in 6x SSC plus 1% SDS. The membranes were air-dried and autoradiographed by exposing to X-ray film (FUJI RX) with an intensifying screen. A positive clone pK-12 strain was separated.

The clone pK-12 was not long enough to encode the entire esterase. The nucleotide sequence of pK-12 was determined by the dideoxy method and a part of the sequence was used to probe a DNA library prepared by digesting chromosomal DNA with *EcoRI* and ligating the fragments to *EcoRI*-digested vector pUC19. By the colony hybridization, a positive clone pEH-16 was isolated. Plasmid pAGE-1, which containing the whole translational region encoding esterase, was constructed by removing an *EcoRI* fragment from pK-12 and ligating said fragment to *EcoRI* site of pEH-16. The construction of plasmid pAGE-1 is shown in Figure 2.

### 3. Sequence Analysis of Esterase Gene and Construction of Restriction Map

A restriction map of the cloned DNA obtained in the above 2. was prepared as follows.

*E. coli* JM109 each transformed with plasmid pK1 and pEH16 was grown and plasmid DNA was prepared according to the method of Birnboim-Doly. Plasmid DNA was digested with various restriction enzymes and the length of each DNA fragment was determined by 1% agarose gel electrophoresis and 5% polyacrylamide gel electrophoresis. Comparing the length of strands of resultant DNA fragments, a restriction map was completed.

### 4. Determination of Nucleotide Sequence of DNA Encoding Esterase and Deduced Amino Acid Sequence

The sequence of 2,175 base pairs of an insert in the plasmid pAGE-1 was determined by the dideoxy method using forward and reverse primer DNAs (Takara Shuzo) for pUC plasmid, successively synthesized primer DNAs and 7-deaza sequencing kit (Takara Shuzo). The over all 2,174 bp nucleotide sequence of an insert in pAGE-1 is provided in Figure 1 and Sequence Listing (SEQ ID No.2). An investigation into the base sequence revealed that there is unique open-reading frame (nucleotide 211 (in GTG) to 1335). Additionally, amino acid sequence encoded by the nucleotide sequence down stream of the possible translational initiation codon of said frame is consistent with that of the N-terminal sequence of purified esterase peptide, which demonstrates that said region of the sequence is the open reading frame encoding the esterase. Thus, esterase was proved to be a protein of a molecular weight of 39,839 composed of 375 amino acid residues. Deduced amino acid sequence is also provided in Figure 1 and Sequence Listing (SEQ ID No.1).

#### Example 3

#### Construction of Expression Plasmids for Esterase

Plasmid pKK223-4 was prepared by deleting one BamHI restriction site from an expression vector pKK223-3 (Boyer et al, Proc. Natl. Acad. Sci. USA, 80: 21-25 (1983); Farmacia). The pKK223-3 was partially digested with BamHI, blunted with T4 DNA polymerase and religated to give the desired plasmid pKK223-4.

To modify the upstream region of the translational initiation codon of the esterase gene, the following  
5 DNA fragments were synthesized using Applied Biosystems DNA synthesizer (Model 380A).

DNA fragment	Sequence identifier number
PH-22	SEQ ID No.3
PH-21	SEQ ID No.4
ES-01	SEQ ID No.5
ES-02	SEQ ID No.6
ES-13	SEQ ID No.7
ES-11	SEQ ID No.8
ES-12	SEQ ID No.9
ES-21	SEQ ID No.10
ES-22	SEQ ID No.11

Synthesized oligonucleotides fragments PH-21, ES-01, ES-11, ES-21, and ES-13 were 5'-end  
20 phosphorylated and subjected to the ligation and annealing with untreated fragments to give the following  
three double-stranded DNA fragments.

5  
10  
15  
20  
25  
30  
35  
40  
45

PH-22	ES-01		
		GATCCTTTTAAATAAAATC   AGGAGGTAAAA AACGATGGACGCCACAGACCATCGCACCGGGCTT	
		GAAAAAATTATTTTAG TCCTCCATTTT   TTGCTACCTGGGTGCTCTGGTAGCGTGGCCCCGAAGC	
BamHI	PH-21	ES-02	Nsp(7524)V
PH-22	ES-11		
		GATCCTTTTAAATAAAATC   AGGAGGTAAAA ATCGATGGACGCCACAGACCATCGCACCGGGCTT	
		GAAAAAATTATTTTAG TCCTCCATTTT   TAGCTACCTGGGTGCTCTGGTAGCGTGGCCCCGAAGC	
BamHI	ES-13	ES-12	Nsp(7524)V
PH-22	ES-21		
		GATCCTTTTAAATAAAATC   AGGAGGTAAAA ATATGGACGCCACAGACCATCGCACCGGGCTT	
		GAAAAAATTATTTTAG TCCTCCATTTT   TATACCTGGGTGCTCTGGTAGCGTGGCCCCGAAGC	
BamHI	ES-13	ES-22	Nsp(7524)V

The double-stranded DNA fragments were kinased at the both ends. An esterase coding region was isolated from plasmid pAGE-1 by the double digestion with restriction enzymes Nsp(7524) V and HindIII. A ligation reaction was carried out in a mixture containing the isolated esterase-encoding DNA fragment, synthesized DNA fragment and BamHI, HindIII-digested, alkaline phosphatase-treated vector pKK223-4. Thus, expression plasmids pAGE-201, 202 and 203 for transforming *E. coli* host cells, which contain down stream of a *tac* promoter a modified DNA sequence and an esterase gene in this order, were obtained.

#### Example 4

#### Preparation of Stereoselective Esterase

##### 1. Growth of Transformants

The esterase capable of asymmetrically hydrolyzing a racemic ester of chrysanthemumic acid (KCA) was produced by culturing *E. coli* JM109/pAGE-201, 202 or 203. *E. coli* JM109 was transformed with a expression plasmid prepared in Example 3 and cultured in a M9 medium (10.5 g K<sub>2</sub>HPO<sub>4</sub>, 4.5 g KH<sub>2</sub>PO<sub>4</sub>, 1.0 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g sodium citrate, 0.2 g MgSO<sub>4</sub> 7H<sub>2</sub>O, 2.0 g glucose, 2 mg/ml thiamine HCl) at 37 °C to a logarithmic phase, where IPTG (isopropyl thio-β-D-galactoside ) was added to the final concentration of 1 mM to induce the expression of the esterase.

Cells were harvested by centrifugation. SDS-PAGE analysis conducted using a portion of cells gave a main band at molecular weight of 40,000 demonstrating that the esterase was expressed to a high extent in *E. coli* transformed by either of plasmid pAGE-201, 202 or 203.

## 2. Asymmetric Hydrolysis of a Racemic Ethyl Ester by Recombinant *E. coli* JM109 Cells

The *E. coli* cells transformed with plasmid pAGE-201, 202 or 203 was evaluated as to the effect on the asymmetric hydrolysis of a racemic ethyl ester (referred to as KCE) of chrysanthemumic acid (cis:trans = 10:90; (+):- = 50:50). *E. coli* JM109/pAGE-201, 202 or 203 cells obtained from 100 ml culture were suspended into 50 ml of 200 mM glycine/sodium hydroxide buffer (pH 10.0). To the suspension was added 1.0 g of KCE and reacted at 37 °C with stirring at 1,000 rpm for 6 hr and the reaction stopped by the addition of 5.0 ml of 35 % HCl. The resultant KCA product and unreacted KCE were extracted with methyl isobutyl ketone (MIBK) from the acidic mixture. The extract was analyzed by gas chromatography (column: Shinchrom F<sub>51</sub> + H<sub>3</sub>PO<sub>4</sub> (10+1%), 2.6 m, 190 °C) and the hydrolytic efficiency was calculated according to area percentage.

To the organic extract was added 20 ml of 0.01 N NaOH and only KCA was extracted into aqueous layer as a sodium salt. The aqueous solution was again treated with 35% HCl and KCA was extracted with MIBK. The extract was concentrated and dehydrated. The content of four isomers of KCA was analyzed as follows. A portion of the extract was mixed with dicyclohexylcarbodiimide and 3,5-dichloroaniline and allowed to stand for 3 hr at room temperature, which was followed by the analysis by a high-performance liquid chromatography (column: SUMIPAX OA-2100 x 2; mobile phase: n-hexane/1,2-dichloromethane (17:3, v/v); flow rate: 1.5 ml/min, detection: 254 nm). Results are given in Table 1 below.

**Table 1**

Strain	hydrolytic efficiency <sup>(1)</sup> (%)	cis/trans ratio (%)			
		(+)-	(-)	(+)-	(-)-
		cis:-cis:trans:trans			
JM109/pAGE-201	96.3	0:	0:	100:	0
JM109/pAGE-202	100	0:	0:	100:	0
JM109/pAGE-203	89.1	0:	0:	100:	0
JM109	0				

<sup>(1)</sup> Hydrolytic efficiency: the amount of resultant (+)-trans KCA to the amount of (+)-trans KCE in the starting material.

## 3. Asymmetric Hydrolysis of a Racemic Ethyl Ester by Recombinant *E. coli* JM105 Cells

Transformants producing esterase activity were prepared and evaluated in the same manner as above 2 except that *E. coli* JM105 was used as a host cell. *E. coli* JM105 cells were transformed with plasmids

pAGE-201, 202 or 203 and grown in M9 medium at 37 °C by inducing the expression of esterase by the addition of IPTG to a final concentration of 1 mM at the logarithmic phase. Cells were harvested and used for hydrolysis of a racemic ethyl ester of KCE (cis:trans = 10:90; (+):(-) = 50:50) in the same manner as above 2. Hydrolytic efficiency was evaluated by the gas chromatography (column: Shinchrom F<sub>51</sub> + H<sub>3</sub>PO<sub>4</sub> (10+1%), 2.6 m, 190 °C). The hydrolytic efficiency was evaluated according to the area percentage. The content of four isomers of KCA was analyzed by HPLC in the same manner as above 2. Results are given in Table 2 below.

Table 2

Strain	hydrolytic efficiency (%)	cis/trans ratio (%)			
		(+)- cis:	(-)- cis:	(+)- trans:	(-)- trans:
JM105/pAGE-201	55.9	0:	0:	100:	0
JM105/pAGE-202	92.9	0:	0:	100:	0
JM105/pAGE-203	100	0:	0:	100:	0

As is clear from the above Tables 1 and 2, the expression of esterase in transformants was extremely promoted by cloning the gene into *E. coli* cells and changing the promoter and upstream region to a suitable sequence for *E. coli*, whereby the asymmetric hydrolysis of racemic compound was surprisingly improved. Thus, according to the present invention, desired (+)-trans KCA can be produced in high yield. When *Arthrobacter globiformis* IFO-12958, which expresses esterase slightly, was grown and 20 µg of a purified enzyme (corresponding to 2.5 L culture) was used for the hydrolysis of 90KCE (concentration: 2 w/v %; temperature: 40 °C; reaction period: 24 hr), the hydrolytic efficiency was only 6.4 % (Japanese Patent Publication (KOKAI) No. 181788/1989).

## SEQUENCE LISTING

5 SEQ ID NO:1

SEQUENCE LENGTH: 375 amino acids

10 SEQUENCE TYPE: amino acid

TOPOLOGY: linear

MOLECULE TYPE: protein

15 Met Asp Ala Gln Thr Ile Ala Pro Gly Phe Glu Ser Val Ala Glu Leu

5 10 15

20 Phe Gly Arg Phe Leu Ser Glu Asp Arg Glu Tyr Ser Ala Gln Leu Ala

20 25 30

Ala Tyr His Arg Gly Val Lys Val Leu Asp Ile Ser Gly Gly Pro His

35 40 45

25 Arg Arg Pro Asp Ser Val Thr Gly Val Phe Ser Cys Ser Lys Gly Val

50 55 60

30 Ser Gly Leu Val Ile Ala Leu Leu Val Gln Asp Gly Phe Leu Asp Leu

65 70 75 80

Asp Ala Glu Val Val Lys Tyr Trp Pro Glu Phe Gly Ala Glu Gly Lys

85 90 95

35 Ala Thr Ile Thr Val Ala Gln Leu Leu Ser His Gln Ala Gly Leu Leu

100 105 110

40 Gly Val Glu Gly Gly Leu Thr Leu Ala Glu Tyr Asn Asn Ser Glu Leu

115 120 125

Ala Ala Ala Lys Leu Ala Gln Met Arg Pro Leu Trp Lys Pro Gly Thr

130 135 140

45 Ala Phe Gly Tyr His Ala Leu Thr Ile Gly Val Phe Met Glu Glu Leu

145 150 155 160

50 Cys Arg Arg Ile Thr Gly Ser Thr Leu Gln Glu Ile Tyr Glu Gln Arg

165 170 175

55

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Ile Arg Ser Val Thr Gly Ala His Phe Phe Leu Gly Leu Pro Glu Ser  
180 185 190

5 Glu Glu Pro Arg Tyr Ala Thr Leu Arg Trp Ala Ala Asp Pro Ser Gln  
195 200 205

10 Pro Trp Ile Asp Pro Ala Ser His Phe Gly Leu Ser Ala Asn Ser Ala  
210 215 220

15 Val Gly Asp Ile Leu Asp Leu Pro Asn Leu Arg Glu Val Arg Ala Ala  
225 230 235 240

Gly Leu Ser Ser Ala Ala Gly Val Ala Ser Ala Glu Gly Met Ala Arg  
20 245 250 255

Val Tyr Ala Ala Ala Leu Thr Gly Leu Ala Ala Asn Gly Asp Arg Ala  
260 265 270

25 Ala Val Ala Pro Leu Leu Ser Glu Glu Thr Ile Gln Thr Val Thr Ala  
275 280 285

30 Glu Gln Val Phe Gly Ile Asp Arg Val Phe Gly Glu Thr Ser Cys Phe  
290 295 300

Gly Thr Val Phe Met Lys Ser His Ala Arg Ser Pro Tyr Gly Ser Tyr  
35 305 310 315 320

Arg Ala Phe Gly His Asp Gly Ala Ser Ala Ser Leu Gly Phe Ala Asp  
40 325 330 335

Pro Val Tyr Glu Leu Ala Phe Gly Tyr Val Pro Gln Gln Ala Glu Pro  
340 345 350

45 Gly Gly Ala Gly Cys Arg Asn Leu Glu Leu Ser Ala Ala Val Arg Lys  
355 360 365

50 Ala Val Thr Glu Leu Ala Gln  
370 375

55

SEQ ID NO:2

SEQUENCE LENGTH: 1125 base pairs

SEQUENCE TYPE: nucleic acid

STRANDNESS: double

TOPOLOGY: linear

MOLECULE TYPE: genomic DNA

ORIGINAL SOURCE:

ORGANISM: *Arthrobacter globiformis*

STRAIN: SC-6-98-28 (FERM BP-3618)

## FEATURES:

NAME/KEY: CDS

LOCATION: from 1 to 1125

IDENTIFICATION METHOD: E

GTGGATGCAC AGACGATTGC CCCTGGATTG GAATCAGTCG CCGAACTCTT TGGCCGTTTC 60  
CTGAGCGAAG ACCGGGAATA TTCAGCCCAG CTCGCGGCCT ACCACCGCGG AGTCAAGGTA 120  
TTGGACATCA GCGGTGGGCC GCACCGCCGC CCGGATTCCG TGACCGGTGT TTTCTCCTGC 180  
TCCAAGGGAG TATCCGGGCT GGTATCGCA CTTTGTGTCC AGGACGGCTT CCTCGACCTC 240  
GACGCCGAAG TGGTCAAGTA CTGGCCGAA TTCGCGCCG AAGGAAAGGC CACGATTACC 300  
GTGGCCCAGC TGCTCTCCCA CCAGGCCGGG CTTCTGGGAG TCGAAGGCGG ACTCACCTC 360  
GCGGAATACA ACAACTCCGA ACTGGCCGCC GCCAAGCTCG CGCAGATGCG GCCGCTGTGG 420  
AAGCCCGGGA CCGCCTTCGG GTACCAGCCC CTGACCATCG GCGTCTTCAT GGAGGAGCTT 480  
TGCCGCCGGA TCACCGGGTC CACGCTCCAG GAAATCTACG AACAGCGGAT CCGCTCGGTC 540  
ACGGGGCCCC ACTTCTTCCT GGGACTGCCT GAGTCCGAGG AACCCCGCTA TGCCACCCTC 600  
CGTTGGGCTG CAGACCCCTC CCAGCCGTGG ATTGATCCCG CCAGCCATTT CGGCCTTTCC 660  
GCAAACCTCG CCGTGGGGGA CATCCTTGAC CTGCCCAACC TCCGCGAGGT CCGCGCAGCC 720  
GGCCTGAGTT CAGCCGCCGG AGTCGCCAGC GCGGAAGGCA TGGCCCGCGT CTACGCTGCG 780  
GCACTCACCG GACTTGCCGC CAACGGCGAC CGAGCCGCCG TCGCGCCCTT CCTCAGCGAA 840  
GAGACCATCC AAACCGTCAC GGCCGAGCAG GTCTTCGGCA TCGACCGGGT GTTCGGCGAG 900

5 ACGAGCTGCT TTGGGACAGT GTTCATGAAA TCGCATGCAC GCTCGCCTTA TGGCAGCTAC 960  
 CGGGCGTTCC GGCACGACGG CGCCAGCCCA TCTTTGGGGT TCGCTGACCC TGTGTATGAA 1020  
 CTCGCCTTCC GGTACGTGCC GCAACAGGCC GAGCCGGGCG GAGCGGGATG CCGCAACCTT 1080  
 10 GAGCTGAGCG CCGCCGTGCG GAAGGCAGTC ACCGAACTGG CTCAG 1125

SEQ ID NO:3

SEQUENCE LENGTH: 20 base pairs

SEQUENCE TYPE: nucleic acid

STRANDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid, synthetic DNA

25 GATCCTTTTT TAATAAAATC 20

SEQ ID NO:4

SEQUENCE LENGTH: 27 base pairs

SEQUENCE TYPE: nucleic acid

STRANDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid, synthetic DNA

40 TTTTACCTCC TGATTTTATT AAAAAAG 27

SEQ ID NO:5

SEQUENCE LENGTH: 44 base pairs

SEQUENCE TYPE: nucleic acid

STRANDNESS: single

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid, synthetic DNA

AGGAGGTAAA AAACGATGGA CGCACAGACC ATCGCACCGG GCTT

34

5 SEQ ID NO:6  
SEQUENCE LENGTH: 35 base pairs  
SEQUENCE TYPE: nucleic acid

10

STRANDNESS: single

15 TOPOLOGY: linear  
MOLECULE TYPE: Other nucleic acid, synthetic DNA  
CGAAGCCCGG TCGGATGGTC TGTGCGTCCA TCGTT

20

35

SEQ ID NO:7

25 SEQUENCE LENGTH: 27 base pairs  
SEQUENCE TYPE: nucleic acid

30

STRANDNESS: single

TOPOLOGY: linear

35 MOLECULE TYPE: Other nucleic acid, synthetic DNA  
TTTTTCCTCC TGATTTTATT AAAAAAG

27

40

SEQ ID NO:8

SEQUENCE LENGTH: 44 base pairs

45 SEQUENCE TYPE: nucleic acid

STRANDNESS: single

50

TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid, synthetic DNA

55 AGGAGGAAAA AATCGATGGA CGCACAGACC ATCGCACCGG GCTT

44

SEQ ID NO:9

5 SEQUENCE LENGTH: 35 base pairs  
SEQUENCE TYPE: nucleic acid

10 STRANDNESS: single  
TOPOLOGY: linear

15 MOLECULE TYPE: Other nucleic acid, synthetic DNA

CGAAGCCCGG TCGGATGGTC TGTGCGTCCA TCGAT 35

20 SEQ ID NO:10

SEQUENCE LENGTH: 42 base pairs  
25 SEQUENCE TYPE: nucleic acid

30 STRANDNESS: single  
TOPOLOGY: linear

MOLECULE TYPE: Other nucleic acid, synthetic DNA

35 AGGAGGAAAA AATATGGACG CACAGACCAT CGCACCGGGC TT 42

40 SEQ ID NO:11

SEQUENCE LENGTH: 33 base pairs  
SEQUENCE TYPE: nucleic acid

45 STRANDNESS: single  
TOPOLOGY: linear

50 MOLECULE TYPE: Other nucleic acid, synthetic DNA

CGAAGCCCGG TCGGATGGTC TGTGCGTCCA TAT 33

55

# Claims

1. An isolated gene encoding an esterase capable of asymmetrically hydrolysing racemic ester of chrysanthemumic acid or its derivative.
- 5 2. The gene of Claim 1, wherein said gene encodes an amino acid sequence presented in the Sequence Listing, SEQ ID No.1.
3. The gene of Claim 1, wherein the DNA molecule comprises the nucleotide sequence presented in the Sequence Listing, SEQ ID No.2.
- 10 4. The gene of Claim 1, wherein the gene is originated from Arthrobacter globiformis SC-6-98-28.
5. A plasmid which contains a DNA of Claim 3.
- 15 6. Plasmid pAGE-201.
7. Plasmid pAGE-202.
8. Plasmid pAGE-203.
- 20 9. A microorganism transformed by a plasmid of Claim 6, 7 or 8.
10. The microorganism of Claim 9 which is Escherichia coli.
- 25 11. The microorganism of Claim 9 which is Escherichia coli JM109.
12. E. coli JM109/pAGE-201.
13. E. coli JM109/pAGE-202.
- 30 14. E. coli JM109/pAGE-203.
15. The microorganism of Claim 9 which is Escherichia coli JM105.
- 35 16. E. coli JM105/pAGE-201.
17. E. coli JM105/pAGE-202.
18. E. coli JM105/pAGE-203.
- 40 19. A process for producing an esterase useful for the asymmetric hydrolysis, which comprises culturing the transformed microorganism of Claim 9, 10, 11, 12, 13, 14, 15, 16, 17 or 18 in an appropriate medium until a sufficient amount of esterase is produced.
- 45 20. A process for asymmetrically hydrolyzing a racemic ester of chrysanthemumic acid or its derivatives to produce (+)-trans acid, which comprises by contacting a culture obtained according to the process of Claim 19 or purified esterase from the culture to the ester to be hydrolyzed.

50

55

Fig. 1 (a)

GGTACCGCTA CCTTTTCATG CACCCCAGCG GTGAGGACCT GAAATTCCTG TCACGCCTGG 60  
 TTTCCGAGGG GAAACTGCAA CCCGTGGTGG ACAGCAGCTA TCCGCTCGAA AAGATCGGGC 120  
 ACGTTTCGCC GCGCTGGAGC AGGGACGCGC CAAGGGCAAG ATCGTGGTGA CCATGGACAC 180  
 GCGCGGCAGT TAGGCAGTTA GGCTGTCCGG GTG GAT GCA CAG ACG ATT GCC CCT 234  
 Met Asp Ala Gln Thr Ile Ala Pro  
 5  
 GGA TTC GAA TCA GTC GCC GAA CTC TTT GGC CGT TTC CTG AGC GAA GAC 282  
 Gly Phe Glu Ser Val Ala Glu Leu Phe Gly Arg Phe Leu Ser Glu Asp  
 10 15 20  
 CGG GAA TAT TCA GCC CAG CTC GCG GCC TAC CAC CGC GGA GTC AAG GTA 330  
 Arg Glu Tyr Ser Ala Gln Leu Ala Ala Tyr His Arg Gly Val Lys Val  
 25 30 35 40  
 TTG GAC ATC AGC GGT GGG CCG CAC CGC CGC CCG GAT TCC GTG ACC GGT 378  
 Leu Asp Ile Ser Gly Gly Pro His Arg Arg Pro Asp Ser Val Thr Gly  
 45 50 55  
 GTT TTC TCC TGC TCC AAG GGA GTA TCC GGG CTG GTC ATC GCA CTT TTG 426  
 Val Phe Ser Cys Ser Lys Gly Val Ser Gly Leu Val Ile Ala Leu Leu  
 60 65 70  
 GTC CAG GAC GGC TTC CTC GAC CTC GAC GCC GAA GTG GTC AAG TAC TGG 474  
 Val Gln Asp Gly Phe Leu Asp Leu Asp Ala Glu Val Val Lys Tyr Trp  
 75 80 85  
 CCG GAA TTC GGC GCC GAA GGA AAG GCC ACG ATT ACC GTG GCC CAG CTG 522  
 Pro Glu Phe Gly Ala Glu Gly Lys Ala Thr Ile Thr Val Ala Gln Leu  
 90 95 100  
 CTC TCC CAC CAG GCC GGG CTT CTG GGA GTC GAA GGC GGA CTC ACC CTC 570  
 Leu Ser His Gln Ala Gly Leu Leu Gly Val Glu Gly Gly Leu Thr Leu  
 105 110 115 120

Fig. 1 (b)

GCG GAA TAC AAC AAC TCC GAA CTG GCC GCC GCC AAG CTC GCG CAG ATG	618
Ala Glu Tyr Asn Asn Ser Glu Leu Ala Ala Ala Lys Leu Ala Gln Met	
125 130 135	
CGG CCG CTG TGG AAG CCC GGG ACC GCC TTC GGG TAC CAC GCC CTG ACC	666
Arg Pro Leu Trp Lys Pro Gly Thr Ala Phe Gly Tyr His Ala Leu Thr	
140 145 150	
ATC GGC GTC TTC ATG GAG GAG CTT TGC CGC CGG ATC ACC GGG TCC ACG	714
Ile Gly Val Phe Met Glu Glu Leu Cys Arg Arg Ile Thr Gly Ser Thr	
155 160 165	
CTC CAG GAA ATC TAC GAA CAG CGG ATC CGC TCG GTC ACG GGC GCC CAC	762
Leu Gln Glu Ile Tyr Glu Gln Arg Ile Arg Ser Val Thr Gly Ala His	
170 175 180	
TTC TTC CTG GGA CTG CCT GAG TCC GAG GAA CCC CGC TAT GCC ACC CTC	810
Phe Phe Leu Gly Leu Pro Glu Ser Glu Glu Pro Arg Tyr Ala Thr Leu	
185 190 195 200	
CGT TGG GCT GCA GAC CCC TCC CAG CCG TGG ATT GAT CCC GCC AGC CAT	858
Arg Trp Ala Ala Asp Pro Ser Gln Pro Trp Ile Asp Pro Ala Ser His	
205 210 215	
TTC GGC CTT TCC GCA AAC TCG GCC GTG GGG GAC ATC CTT GAC CTG CCC	906
Phe Gly Leu Ser Ala Asn Ser Ala Val Gly Asp Ile Leu Asp Leu Pro	
220 225 230	
AAC CTC CGC GAG GTC CGC GCA GCC GGC CTG AGT TCA GCC GCC GGA GTC	954
Asn Leu Arg Glu Val Arg Ala Ala Gly Leu Ser Ser Ala Ala Gly Val	
235 240 245	
GCC AGC GCG GAA GGC ATG GCC CGC GTC TAC GCT GCG GCA CTC ACC GGA	1002
Ala Ser Ala Glu Gly Met Ala Arg Val Tyr Ala Ala Ala Leu Thr Gly	
250 255 260	

Fig. 1 (c)

CTT GCC GCC AAC GGC GAC CGA GCC GCC GTC GCG CCC CTC CTC AGC GAA	1050
Leu Ala Ala Asn Gly Asp Arg Ala Ala Val Ala Pro Leu Leu Ser Glu	
265                      270                      275                      280	
GAG ACC ATC CAA ACC GTC ACG GCC GAG CAG GTC TTC GGC ATC GAC CGG	1098
Glu Thr Ile Gln Thr Val Thr Ala Glu Gln Val Phe Gly Ile Asp Arg	
285                      290                      295	
GTG TTC GGC GAG ACG AGC TGC TTT GGG ACA GTG TTC ATG AAA TCG CAT	1146
Val Phe Gly Glu Thr Ser Cys Phe Gly Thr Val Phe Met Lys Ser His	
300                      305                      310	
GCA CGC TCG CCT TAT GGC AGC TAC CGG GCG TTC GGG CAC GAC GGC GCC	1194
Ala Arg Ser Pro Tyr Gly Ser Tyr Arg Ala Phe Gly His Asp Gly Ala	
315                      320                      325	
AGC GCA TCT TTG GGG TTC GCT GAC CCT GTG TAT GAA CTC GCC TTC GGG	1242
Ser Ala Ser Leu Gly Phe Ala Asp Pro Val Tyr Glu Leu Ala Phe Gly	
330                      335                      340	
TAC GTG CCG CAA CAG GCC GAG CCG GGC GGA GCG GGA TGC CGC AAC CTT	1290
Tyr Val Pro Gln Gln Ala Glu Pro Gly Gly Ala Gly Cys Arg Asn Leu	
345                      350                      355                      350	
GAG CTG AGC GCC GCC GTG CGG AAG GCA GTC ACC GAA CTG GCT CAG	1335
Glu Leu Ser Ala Ala Val Arg Lys Ala Val Thr Glu Leu Ala Gln	
365                      370                      375	
TAGGATGAAG CATGACCGAA CCGAGATTCA CCGTTGAAAC AGCCATGGTC CTTGCCGAAG	1395
TGGCGCACAA TCGCCAGAAG GACAAGCTGA AGCGGCCCGTA CCGCGAGCAC GTTCTGSCGG	1455
TAGGGGATGC GCTCGCCGAC TTCGACGACG ACATCCGGAT CGCAGGTTAC CTGCACGACA	1515
TGCCCAAGGA CACGCCGATT ACCAAGCAGG CGCTGCTCGA CATGGGTGTT TCCGAGCGTG	1575
CCGTGGGCAT CATCGAACGC GTGACGCGCC GATTCCAGCA TGATTCCGAC AACGATGAAG	1635
CCGTGTTTCT GCATATCGCA CAGGACCATG ACGCCACCCT GGTCAAGATC GCCTGCAACG	1695

Fig. 1 (d)

CCCACAACCTC CGTGCCGGAG CGCGTGCGGG CGCTCGCCGA AAAGTGGCCG GACAAGGCAC 1755  
CCAGCACGCG CTACGCAGAC GCCCCGGGAAG TGCTCTACAA GGCGGTGCCA CGGGGTGAAA 1815  
TCCACTTGGT CCTGCAACGC ATCAACCCTG ATCTGCTGTC CGAGATGGAC CGGCTGGCGG 1875  
ACTAGTAGTT CGACGCGGCA GGTCTCGCCT GTCTACGGTT TCGTCGGTTC GTCCTGAATC 1935  
GGGTTGGCAG CGGCCGCCGC CTCCCGGGTA GCGGATGCGG TGACGTCCGC GAGAATCCGT 1995  
TGGTGGATCT GGGCGGTGAG CTCGTGAATC GCACGCGTCA GGTCGGTGTT CTCCTCGAGC 2055  
AGATGCTCCT GGGCGTTGTA GTCATGGTCT GCCTTCACCT GCTGGAAAGC CGCCTGACGG 2115  
TTCTGTCCGA TCATGACGAA GGTCGAGAGG AAGATCGCTT CGAGCGAGAC GATCAAGGT 2175

Fig. 2

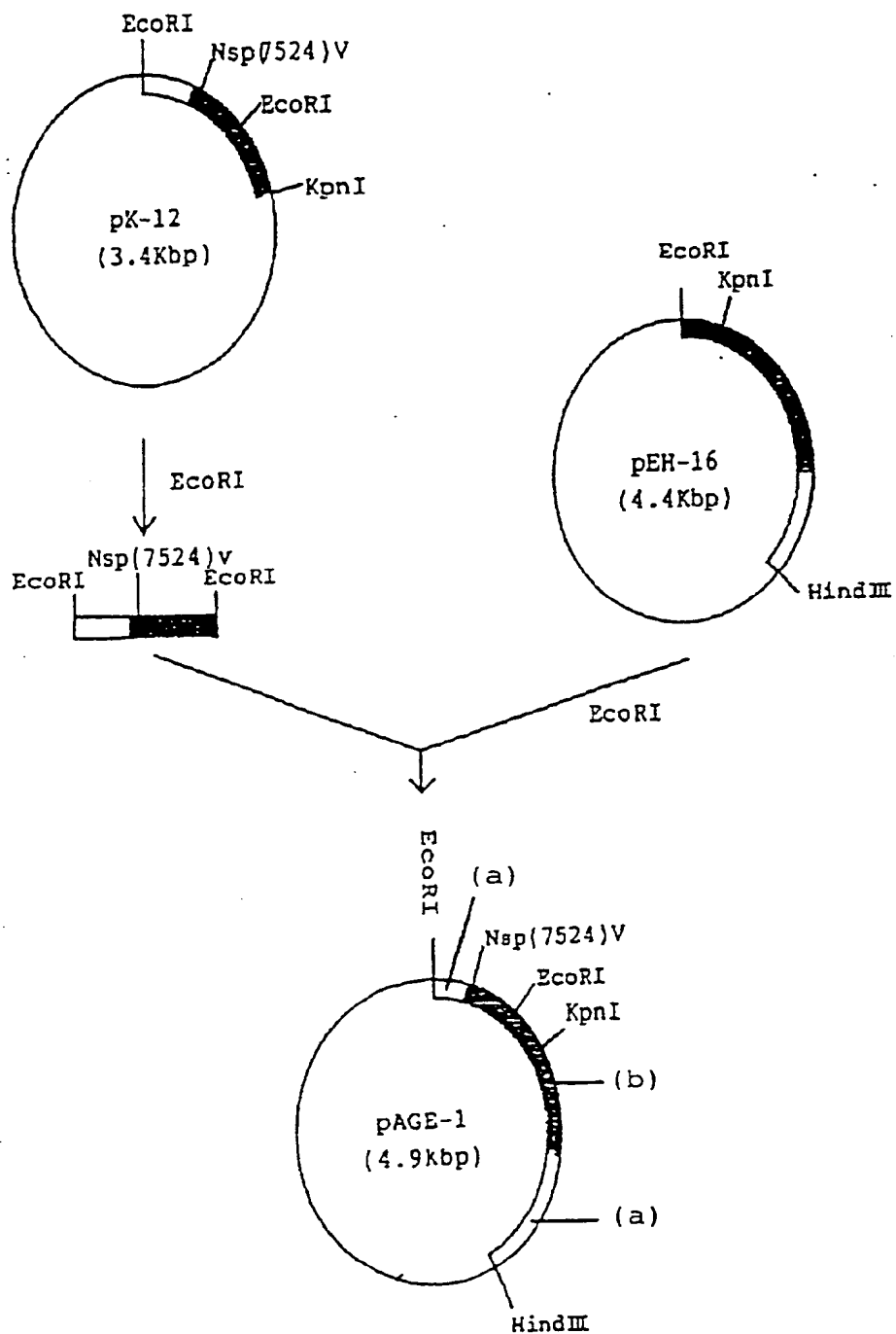
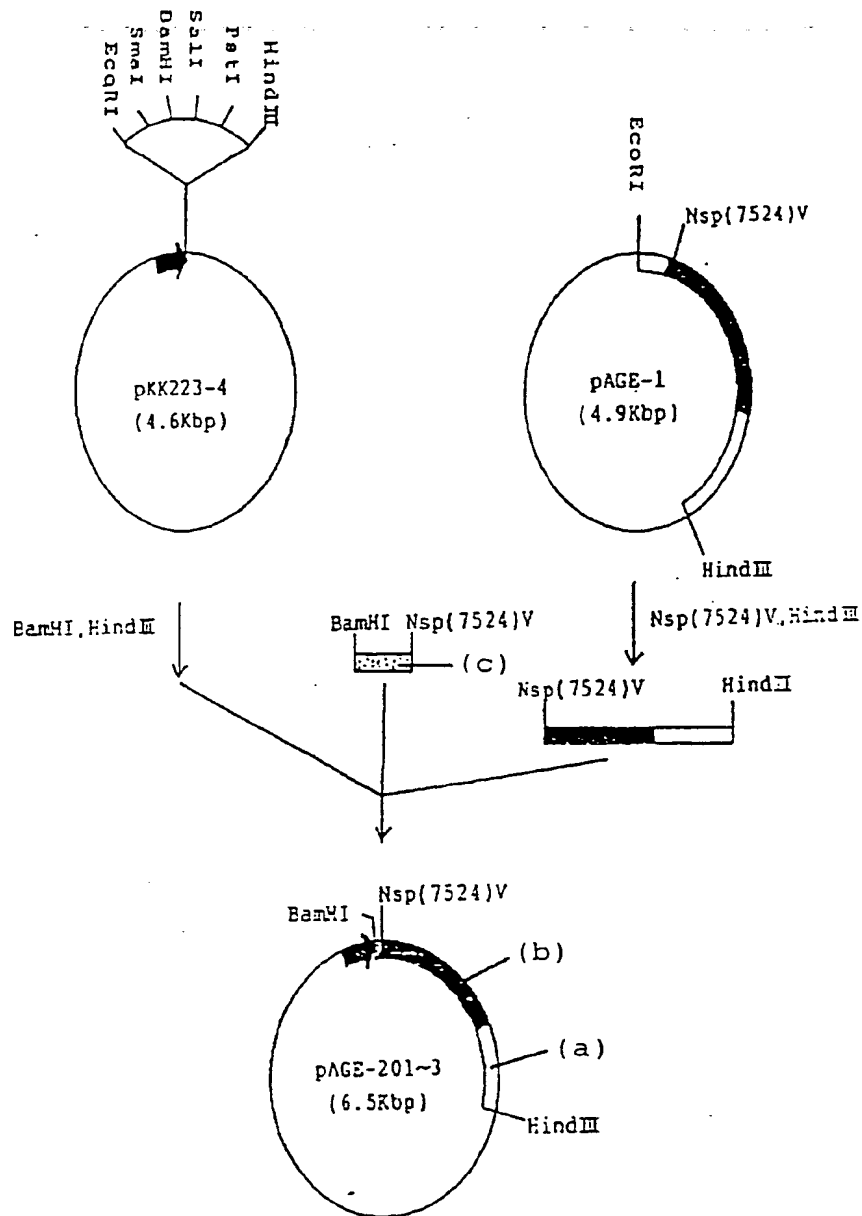


Fig. 3



(19)



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(54) **Gene encoding asymmetrically active esterase.**

(57) An isolated gene encoding an esterase capable of asymmetrically hydrolysing an ester of chrysanthemumic acid or its derivative to give an intermediate useful for the production of pharmaceutically and/or agriculturally useful compounds, expression plasmids containing said gene, microorganisms transformed with said expression plasmids, and the production of the esterase by culturing said transformants.

**EP 0 497 103 A3**



European Patent  
Office

## EUROPEAN SEARCH REPORT

Application Number

EP 92 10 0271

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
A	EP-A-0 264 457 (SUMITOMO) 27 April 1988 * claims; examples 34,37 *	1	C12N15/55 C12N9/18 C12N1/20 C12P41/00 C12P7/40 /(C12N1/21, C12R1:19)
A	CHEMICAL ABSTRACTS, vol. 112, no. 13, 26 March 1990, Columbus, Ohio, US; abstract no. 117365v, SUGIMOTO, M. ET AL. 'Novel esterase manufacture with Arthrobacter.' page 610 ; * abstract * & JP-A-1 181 788 (SUMITOMO) 19 July 1989	1	
A	EP-A-0 299 558 (GIST-BROCADES) 18 January 1989 * claims *	1	
E	CHEMICAL ABSTRACTS, vol. 118, no. 3, 18 January 1993, Columbus, Ohio, US; abstract no. 21081x, MITSUTA, MASARU ET AL. 'Arthrobacter mutant for enzymic resolution of racemic chrysanthemic acid ester.' page 583 ; * abstract * & JP-A-4 234 991 (SUMITOMO) 24 August 1992	1	
			TECHNICAL FIELDS SEARCHED (Int. Cl.5)
			C12N C12P C12R
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 20 APRIL 1993	Examiner DELANGHE L.L.M.
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons ----- & : member of the same patent family, corresponding document	

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